Exhibit I

FHX 7-31-95#3

Comparison of the immune response to recombinant gp120 in humans and chimpanzees

Phillip W. Berman, Donna J. Eastman, Denise M. Wilkes, Gerald R. Nakamura, Timothy J. Gregory*, David Schwartz†, Geoffrey Gorse‡, Robert Belshe‡, Mary Lou Clements† and Randal A. Byrn§

Objective: To assess similarities and differences in antibody responses to recombinant (r) HIV-1_{IIIB} gp120 in chimpanzees, previously protected from HIV-1 infection, and human volunteers immunized in connection with a Phase I clinical trial.

Methods: Frozen sera from humans immunized with rgp120 from HIV-1_{IIIB} and chimpanzees immunized with the same antigen or recombinant soluble gp160 were compared in a variety of serologic assays.

Results: The magnitude of the antibody response to gp120 was similar in both species; however, the half-life of the antibody response to rgp120 was approximately 4.5 times longer in humans (9 weeks) than in chimpanzees (2 weeks). Antibodies to gp120 in both species were broadly cross-reactive with gp120 from diverse isolates of HIV-1 and were effective in blocking the binding of gp120 to CD4. Antibody binding to native gp120 was greater than to denatured gp120 in both species. Antibody responses to the principal neutralizing determinant (V3 domain) and virus neutralization titers were approximately 10-fold lower in humans than chimpanzees. The relative avidity of antibody binding to gp120 was higher in the sera from the immunized chimpanzees than in the immunized humans.

Conclusions: While the antibody responses to rgp120 elicited in man and chimpanzees were in many ways similar, significant differences did occur. Predictions made on the basis of chimpanzee immunogenicity studies over-estimated the potency of the virus neutralizing titers and under-estimated the duration of the antibody response achieved in humans.

AIDS 1994, 8:591–601

Keywords: HIV-1, vaccine, chimpanzees, gp120

Introduction

Because of their unique susceptibility to HIV-1 infection, chimpanzees have been used in preclinical efficacy studies to test candidate HIV-1 vaccines [1–6]. HIV-1 infection in chimpanzees differs from that in humans because it does not appear to be pathogenic [7,8]. Thus, animals that have been infected for many years show no signs of CD4 cell depletion, lymphadenopathy, decreased immune function, or the increased susceptibility to opportunistic infections that are the hallmarks of HIV-1 infection in humans [9]. In previous studies we demonstrated that a vaccine consisting of a recombinant gp120, derived from the IIIB strain of HIV-1 (IIIB-rgp120), formulated in aluminum hydroxide (alum) adjuvant, was able to protect chimpanzees from experimental infection with the homologous (HIV-1_{IIIB}) virus [3].

From the Departments of Immunology and *Process Sciences, Genentech Inc., South San Francisco, California, the †Center for Immunization Research, Johns Hopkins University, Baltimore, Maryland, the ‡Division of Infectious Diseases, St Louis University School of Medicine, St Louis, Missouri and the §Department of Hematology and Oncology, New England Deaconess Hospital, Harvard Medical School, Boston, Massachusetts, USA.

Sponsorship: Supported in part by NIAID contracts NO1-AI-05064, and NO1AI05061.

Requests for reprints to: Phillip W. Berman, Department of Immunology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080, USA.

Date of receipt: 25 October 1993; revised: 7 February 1994; accepted: 14 February 1994.

The IIIB-rgp120 vaccine, produced in Chinese hamster ovary (CHO) cells, was fully glycosylated [10] and folded in a functionally relevant conformation as determined by its disulfide structure [10] and its ability to bind to CD4 with high affinity [11]. A recently completed double-blind, placebo controlled Phase I clinical trial [National Institutes of Allergy and Infectious Diseases (NIAID AIDS Vaccine Evaluation Group (AVEG Study: 006)] [12] provided data on the safety and immunogenicity of IIIB-rgp120 in humans and provided the first opportunity to directly compare the immune responses to IIIB-rgp120 in humans and chimpanzees.

Although gp120, V3 domain, CD4 blocking and virus neutralization antibody titers were determined in both chimpanzee [3] and human [12] IIIB-rgp120 immunogenicity studies, the results could not be directly compared because different assay formats were used. In the present studies, antisera from both studies were re-analyzed using the same assays in order to directly compare the immune response to IIIB-rgp120 in chimpanzees and humans. In addition to the four assays described above, the antisera from both studies were analyzed, for the first time, in several other serologic assays. The breadth of the immune response to IIIB-rgp120 was observed in enzyme-linked immunosorbent assays (ELISA) where antibody binding to a panel of recombinant gp120 from seven diverse isolates of HIV-1 was measured. Similarly, antibody binding to a panel of synthetic peptides derived from the V3 domains of eight diverse isolates of HIV-1 was measured. Other assays comparing the binding of antisera to native and denatured IIIB-gp120 and the relative avidity of antibody binding to IIIB-rgp120 are described for the first time. The results obtained highlight the similarities and differences in the immune responses of chimpanzees and humans to IIIB-rgp120, and better define the role that chimpanzee data can play in preclinical immunogenicity studies of candidate HIV-1 vaccines.

Materials and methods

Experimental design

Twenty-eight volunteers were randomly assigned to one of three treatment groups; 10 subjects received 100 µg IIIB-rgp120 in alum adjuvant, 10 received 300 µg IIIB-rp120 in alum adjuvant, and eight received alum adjuvant alone as a placebo control. All subjects received three injections given at 0,1, and 8 months. A detailed description of the enrollment criteria, including the age, sex, and racial composition of the volunteers, and the safety monitoring procedures has been published previously [12]. The immunization schedule utilized was designed to duplicate, as closely as possible, the schedule that was used previously to elicit protective immunity in chimpanzees [3]. Blood was collected at multiple intervals and serum and peripheral blood mononuclear cells (PBMC) were evaluated in a number of immunogenicity assays. Immunogenicity and safety data generated in the NIAID AVEG Central Immunology Laboratory has been presented elsewhere [12]. The serologic data presented here were derived from assays performed at Genentech, Inc., (South San Francisco, California, USA), using frozen serum specimens from both chimpanzee [3] and human immunization studies [12] described previously.

Antigens

IIIB-rgp120 and rgp120 from the MN strain of HIV-1 (MN-rgp120) were produced in CHO cells and prepared as described previously [10,13,14]. A soluble (s) recombinant form of gp160 from the IIIB strain of HIV-1 (IIIB-rsgp160) was also prepared as described previously [3]. rgp120 from the NY-5 (NY-5-rgp120), the Z6 (Z6-rgp120), the Z321(Z321-rgp120), the JRcsf (JRcsf-rgp120), and the A244 (A244-rgp120) strains of HIV-1 were produced as described previously [14,15]. A 24 amino-acid synthetic peptide, NNTRKSIRIQRGPGRAFVTIGKIG, from the V3 domain of IIIB-gp120 was provided by Clifford Quan (Genentech Inc.). V3 domain peptides from various other HIV-1 isolates were purchased from American Bio-Technologies, Inc., (Cambridge, Massachusetts, USA). Denatured gp120 was prepared by chemical reduction and carboxymethylation as described previously [15].

Antisera and serologic assays

Sera from chimpanzees that received three 300 µg doses of IIIB-rgp120, IIIB-rsgp160, or alum placebo were collected as described previously [3] and stored frozen at −20°C. Sera from human volunteers immunized with either 100 µg of IIIB-rgp120, 300 µg of IIIB-rgp120, or alum adjuvant placebo were collected as described previously [12] and stored at −20°C. Antibody binding to recombinant proteins and synthetic peptides was measured by ELISA as described previously [3,14,15]. The methods used for coating recombinant proteins and synthetic peptides to microtiter dishes, the antibody incubation and washing conditions, and the antibody binding calculations have been described previously [14,15]. End-point dilution titers, calculated from binding curves, were used to quantitatively measure the magnitude of antibody binding to recombinant proteins and synthetic peptides. Antibody binding titers were reported as $\log_{10}$ of the end-point dilution titer. Qualitative measurements of antibody cross-reactivity were obtained by ELISA at single serum dilutions. In these assays optical density (OD) values at 492 nm were reported. For CD4 blocking studies the binding of ^{125}I-labeled IIIB-rgp120 or ^{125}I-labeled MN-rgp120 to cell surface CD4 was measured as described previously [11,14]. Antibodies to native and

denatured gp120 were measured by coating native or reduced and carboxymethylated IIIB-rgp120 on to ELISA plates as described previously [15]. Monoclonal antibodies (MAb) to IIIB-rgp120 or rsgp160, used to validate the native/denatured gp120 binding assay and the antibody binding avidity assays, have been described previously [15]. CD4-immmunoglobulin (Ig) G was prepared as described previously [16].

Avidity assays

The relative avidity of antibody binding to IIIB-rgp120 was determined using the method of Meurman *et al.* [17]. Briefly, IIIB-rgp120 was coated onto microtiter dishes at 2 μg/ml concentrations in 0.1 M sodium carbonate buffer (pH 9.8). The plates were then blocked with phosphate buffered saline [PBS, consisting of 20 mM sodium phosphate, 0.15 M sodium chloride (pH 7.5)] containing 0.5% bovine serum albumin (Type III; Sigma, St Louis, Missouri, USA) followed by a 5-min incubation with an 8 M urea solution. After a final wash with PBS containing 0.1% Tween-20 (PBS-Tween, Sigma), serial dilutions of polyclonal antibodies or MAb (100 μl per well) were added to the coated wells and allowed to incubate at 25°C for 2 h. Parallel sets of wells were washed three times with PBS-Tween alone, or with PBS-Tween containing 8 M urea for 5 min followed by washing with PBS-Tween. After incubation with affinity-purified horseradish peroxidase conjugated goat antibodies to human IgG (Cappel Inc., Malvern, Pennsylvania, USA), and washing, the wells were incubated with the colorimetric substrate, o-phenylenediamine dihydrochloride (Sigma), as described previously [3,15]. Antibody binding titers were measured by calculating the serum dilution that resulted in an OD value twice that obtained for a 1:100 dilution of pooled normal human serum. Antibody binding avidity was calculated for each serum by dividing the mean titers from duplicate measurements from the urea washed wells, by the mean titers obtained from the PBS-Tween washed wells.

Measurement of virus neutralizing antibodies

The ability of antisera to neutralize virus infectivity was measured in a a colorometric MT-2 cell cytotoxicity assay similar to that described by Pawels *et al.* [18]. MT-2 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH (contributed by Dr Douglas Richman). Briefly, a stock of HIV-1_{IIIB} grown in H9 cells was pre-titrated to produce 90% cytopathic effect in MT-2 cells over the course of the assay period. On average, this amount of virus corresponds to 2000 median tissue culture infectious dose ($TCID_{50}$) units. Serial dilutions of antibody or serum were prepared in 50 μl volumes of complete medium and then 50 μl of a prediluted HIV-1 stock was added to each well. After incubation for 1 h at 4°C, MT-2 cells were added (100 μl containing 4×10^5 MT-2 cell/ml) and the plates were incubated for 5 days at 37°C in 5% CO_2. Viable cells were measured using metabolic conversion of the formazan MTT [3(4,5-dimethylthiazole-2-yl)2,5-diphenyl tetrazolium bromide] dye. Each well received 20 μl of a 5 mg/ml MTT solution in PBS. After 4 h incubation at 37°C, the dye precipitate was dissolved by removing 100 μl of the cell supernatant, adding 130 μl of 10% Triton X-100 (United States Biochemical Corp., Cleveland, Ohio, USA) in acid isopropanol, then pipeting until the precipitate was dissolved. OD of the wells was determined at 540 nm. The percentage inhibition was calculated using the formula: 1 – [(virus control experimental)/(virus control medium control)].

Results

Magnitude and duration of the antibody response to IIIB-rgp120

Although antisera from the chimpanzees and human volunteers immunized with IIIB-rgp120 had been assayed previously for antibodies to IIIB-rgp120, the results could not be directly compared because the antisera were tested using two different assays: a radioimmunoprecipitation assay (RIPA) for the chimpanzee sera [3], and a single-point ELISA for the human sera [12]. In this study, antisera from both studies were evaluated in a IIIB-rgp120 specific ELISA [15] where end-point dilution titers were measured. Six of the 10 volunteers in the 100 μg dose group (mean $\log_{10}$ titer, 2.46) and six of 10 volunteers in the 300 μg dose group (mean $\log_{10}$ titer, 2.38) developed antibodies to IIIB-rgp120 after the primary immunization (Table 1). Neither of the two chimpanzees immunized with IIIB-rgp120 developed detectable levels of antibodies to IIIB-rgp120 after the primary immunization; however, one of the chimpanzees immunized with IIIB-rsgp160 exhibited a $\log_{10}$ titer of 2.20 after a single immunization.

After the second immunization (week 4), all of the chimpanzees and all of the humans developed antibodies to IIIB-rgp120 (Fig. 1). The mean $\log_{10}$ end-point dilution titers were 4.01 for the IIIB-rgp120 immunized animals and 4.00 for the IIIB-rsgp160 immunized animals. The humans immunized with the 100 μg dose exhibited mean $\log_{10}$ titer of 3.63 and those receiving the 300 μg dose exhibited mean $\log_{10}$ titer of 3.81 at week 6.

A third immunization was given to the humans and the chimpanzees 32 weeks after the primary immunization. At this time, the antibody levels in the chimpanzees had fallen approximately 100-fold (Fig. 1), whereas the mean antibody titers in the humans receiving both the 100 and 300 μg doses of IIIB-

Table 1. Antibody titers to recombinant gp120 from the IIIB strain of HIV-1 (IIIB-rgp120) and V3 peptide in humans and chimpanzees* immunized with IIIB-rgp120.

	Humans		Chimpanzees	
Mean (SE)	IIIB-rgp120 (100 μg)	IIIB-rgp120 (300 μg)	IIIB-rgp120 (300 μg)	IIIB-rsgp160 (300 μg)
IIIB-rgp120 titers ($\log_{10}$)				
1st Immunization (week 2)	2.46 (0.12)	2.38 (0.03)	< 2.00	2.20
Responders/total	6/10	6/10	0/2	1/2
2nd Immunization (week 6)	3.63 (0.17)	3.81 (0.27)	4.01 (0.67)	4.00 (0.11)
Responders/total	10/10	9/9	2/2	2/2
3rd Immunization (week 34)	4.12 (0.15)	4.67 (0.06)	4.91 (0.36)	4.85
Responders/total	9/9	10/10	2/2	2/2
HIV-1_{IIIB}V3 titers ($\log_{10}$)				
1st Immunization (week 2)	< 1.70	< 1.70	< 1.70	< 1.70
Responders/total	0/10	0/10	0/2	0/2
2nd Immunization (week 6)	2.50	< 1.70	2.23	2.28
Responders/total	1/9	0/9	1/2	1/2
3rd Immunization (week 34)	2.01 (0.07)	2.39 (0.13)	3.43 (0.11)	2.82 (0.12)
Responders/total	5/9	10/10	2/2	2/2
RCM-IIIB-rgp120 titers ($\log_{10}$)				
3rd Immunization (week 34)	3.63 (0.14)	4.12 (0.08)	4.56 (0.28)	4.50 (0.18)
Responders/total	9/9	10/10	2/2	2/2
†Ratio: native/RCM IIIB-rgp120 titers	3.09	3.55	2.24	2.24

*Human volunteers and chimpanzees were given a primary immunization (1st) at week 0, a secondary immunization (2nd) at week 4, and a tertiary immunization (3rd) at week 32. Serum was collected for antibody-binding assays at 2 weeks after each immunization. Data represents the means of the $\log_{10}$ individual titers with SE given in parentheses. †Ratio of arithmetic means (non-log transformed data). RCM, reduced and carboxymethylated.

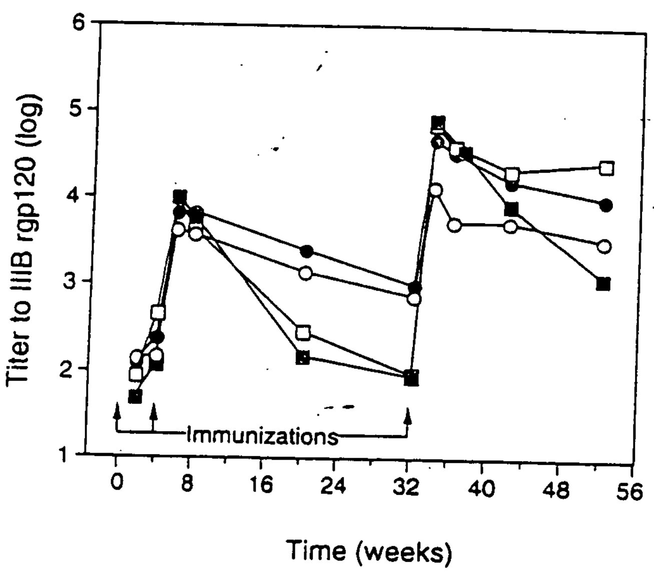

Fig. 1. Magnitude and duration of antibody response to recombinant gp120 from the IIIB strain of HIV-1 (IIIB-rgp120) in humans and chimpanzees. Humans, were immunized with either 100 μg IIIB-rgp120 (O) or 300 μg IIIB-rgp120 (●) according to a 0, 1, and 8-month schedule (arrows). Chimpanzees were immunized with either IIIB-rgp120 (■) or IIIB-rsgp160 (□) according to the same schedule. All antigens were formulated in aluminum hydroxide adjuvant. Serum was collected at various times during the immunization regime and assayed for antibodies to IIIB-rgp120 by enzyme-linked immunosorbent assay. The chimpanzees were challenged with HIV-1_{IIIB} at 3 weeks after the last immunization as described previously [3].

rgp120 had fallen approximately 10-fold. The chimpanzees and all of the humans exhibited booster responses to IIIB-rgp120 after the third immunization (Fig. 1, Table 1). However, the humans receiving the 300 μg dose developed significantly higher antibody titers after the third injection (mean $\log_{10}$ titer, 4.67) than those in the 100 μg dose group (mean $\log_{10}$ titer, 4.12). After receiving the third immunization, the chimpanzees immunized with IIIB-rgp120 developed a mean $\log_{10}$ titer of 4.91 and IIIB-rsgp160 immunized animals developed a mean $\log_{10}$ titer of 4.85. Thus, after the third immunization the chimpanzees and the humans exhibited comparable IIIB-rgp120 titers. For purposes of comparison, the antibody titer to IIIB-rgp120 was measured in a pool of serum prepared from 25 individuals infected with HIV-1. The observation that a $\log_{10}$ titer of 5.05 was measured for this pool (Fig. 2) suggested that immunization with a candidate vaccine could elicit IIIB-rgp120 titers similar to those found in the sera of HIV-1-infected individuals.

There was a striking difference between humans and chimpanzees in the kinetics of the antibody response to IIIB-rgp120. After both the second and third immunizations the decay of the antibody response to IIIB-rgp120 was far more rapid in IIIB-rgp120 immunized chimpanzees than in humans (Fig. 1). Based on the decay curves following the second and third immunizations, the half-life of the antibody response to IIIB-rgp120 in

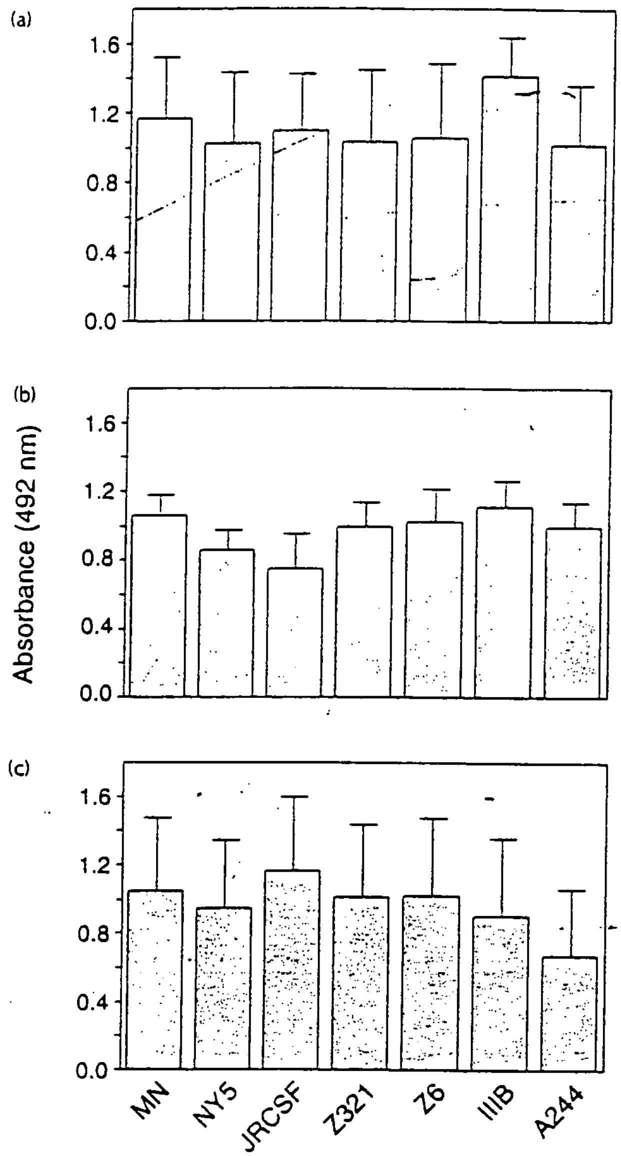

Fig. 2. Relative cross-reactivity of antisera to recombinant gp120 from the IIIB strain of HIV-1 (IIIB-rgp120) with recombinant envelope glycoproteins from diverse isolates of HIV-1. (a) Antisera from chimpanzees (n = 2) or (b) humans (n = 10) that received three 300 µg immunizations with IIIB-rgp120 were assayed by enzyme-linked immunosorbent assay for the ability to bind to rgp120 from seven diverse isolates of HIV-1. (c) The cross-reactivity of serum from HIV-1-infected humans (n = 5) is shown for purpose of comparison. Data represents mean optical density values measured at a single, arbitrarily selected serum dilutions for each group (i.e., 1 : 450 for chimpanzee antisera; 1 : 100 for antisera from IIIB-rgp120 immunized humans; 1 : 900 for antisera from HIV-1-infected humans). Error bars represent standard deviations. X-axes indicate the HIV-1 isolates from which the recombinant proteins were derived.

chimpanzees was calculated to be approximately 2 weeks, whereas the half-life of the antibody response in humans was calculated to be approximately 9 weeks.

Cross-reactivity of antibodies to IIIB-rgp120

An ELISA using a panel of purified rgp120 prepared from seven diverse isolates of HIV-1 [15,19] was used to assess the breadth of the antibody cross-reactivity to IIIB-rgp120 elicited in chimpanzees (Fig. 2a) and humans (Fig. 2b). The panel included envelope glycoproteins from four of the five international subtypes [20]. Thus, rgp120 from the African Z321 isolate represented subtype A; rgp120 from the US isolates MN, IIIB, JRcsf and NY-5 represented subtype B; rgp120 from the African Z6 isolate represented subtype D; and rgp120 from the Northern Thailand isolate A244 represented subtype E. It was found that all of the antisera exhibited significant cross-reactivity, with the binding activity in the IIIB-rgp120 immunized volunteers closely resembling that seen in the immunized chimpanzees. For purposes of comparison, the cross-reactivity of antibodies in the sera of HIV-1-infected humans was also measured (Fig. 2c). As for the IIIB-rgp120 immunized subjects, the sera from HIV-1-infected individuals were broadly cross-reactive and reacted with all recombinant envelope glycoproteins. These data demonstrate that IIIB-rgp120 possesses conserved immunodominant epitopes and is effective in eliciting broadly cross-reactive antibodies to gp120 in both humans and chimpanzees. However, these results represent only a qualitative assessment of antibody cross-reactivity, and do not address the functional significance of quantitative differences seen between individuals in antibody binding to different envelope glycoproteins, when end-point dilution titers are calculated (data not shown).

Antibodies that blocked the binding of gp120 to CD4

As reported previously [12], four of the nine individuals in the 100 µg dose group and all 10 individuals in the 300 µg dose group possessed antibodies that blocked the binding of gp120 to CD4 2 weeks after the third immunization. Antibodies that blocked CD4 binding were present in both of the IIIB-rgp120-injected chimpanzees at this time [3]. The specificity of the CD4-blocking antibodies was investigated in studies where the ability of chimpanzee and human antisera to block the binding of IIIB-rgp120 to cell-surface CD4 was compared with its ability to block the binding of MN-rgp120 to cell-surface CD4. It was found (Fig. 3) that the sera from the IIIB-rgp120-immunized volunteers were always more effective in blocking the binding of the homologous HIV-1_{IIIB}-derived protein to CD4 than in blocking the binding of the heterologous HIV-1_{MN} derived protein. The sera from the IIIB-rgp120 immunized chimpanzees similarly exhibited more po-

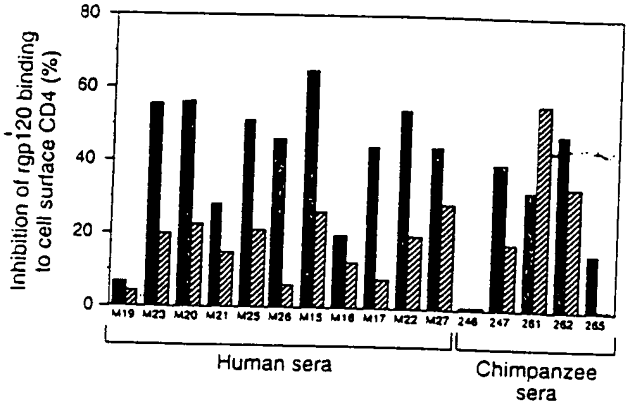

Fig. 3. CD4 blocking activity of chimpanzee and human antisera to recombinant gp120 from the IIIB strain of HIV-1 (IIIB-rgp120). The ability of antisera from humans (1 : 150 dilution) and chimpanzees (1 : 80 dilution) immunized with three 300 µg doses of IIIB-rgp120 to block the binding of either ^{125}I-labeled IIIB-rgp120 (■) or ^{125}I-labeled MN-rgp120 (▨) to cell-surface CD4 was measured as described previously [11]. Data represents the percentage decrease in ^{125}I-rgp120-specific binding to cell surface CD4 in the presence hyperimmune serum collected 2 weeks after the third immunization with IIIB-rgp120. Human serum M19, and chimpanzee serum 246, were obtained from adjuvant immunized placebo controls.

tent CD4 blocking activity to IIIB-rgp120 than to MN-rgp120. However, one of the IIIB-rsgp160 immunized chimpanzees exhibited better CD4 blocking of MN-rgp120 than IIIB-rgp120. These results suggested that both common and strain-specific CD4-blocking antibodies were elicited in chimpanzees and humans immunized with IIIB-rgp120.

Antibodies to epitopes dependent on secondary structure

In order to identify other aspects of the antibody response that might correlate with protective immunity, studies were performed to determine the extent to which the antibody response to IIIB-rgp120 was directed at epitopes dependent on the conformation imposed by disulfide bonding. For this purpose, an ELISA using chemically reduced and carboxymethylated IIIB-rgp120 (RCM IIIB-rgp120), used previously [15] to identify MAb reactive with conformation dependent epitopes, was utilized. To validate this assay, the binding of MAb or rCD4-IgG [16] RCM IIIB-rgp120 was compared with binding to native (properly folded) IIIB-rgp120. It was found that MAb to the V3 domain of IIIB-rgp120 (11G5, 10F6, and 10D8) all exhibited comparable binding to native and RCM IIIB-rgp120 (Table 2). Similarly, the 5B6 MAb specific for an epitope within the 30 amino acid fragment of herpes simplex virus glycoprotein D at the amino terminus IIIB-rgp120 [13], bound equally well to the native and denatured proteins. These experiments demonstrated that similar amounts of native and RCM IIIB-rgp120 were coated onto the microtiter plates. Conversely, MAb 6E10, known to bind to a conformation dependent epitope [15], did not react with the RCM protein but did bind to the native protein. In control experiments, CD4 IgG bound only to the native protein and not to RCM IIIB-rgp120 (Table 2), confirming that the CD4 binding site was dependent on an intact disulfide structure. As expected, antibodies to gp41 failed to bind either protein preparation.

Table 2. Binding of monoclonal antibodies and CD4- immunoglobulin (Ig) G to native and denatured recombinant gp120 from the IIIB strain of HIV-1 (IIIB-rgp120).

Antibody/ receptor	Epitope (residue no.)	Titers: Native IIIB-rgp120	Titers: RCM-IIIB-rgp120
CD4-IgG	Discontinuous	625	<1
5B6	1–25	590	643
6E10	163–200	460	<1
11G5	302–325	1007	1128
10F6	302–325	618	633
10D8	302–325	632	628
14F12	510–683	<1	<1

Titers to native and denatured IIIB-rgp120 were derived from end-point dilution titrations beginnning with 20 µg of each antibody or CD4-IgG, values represent dilutions at one half maximum binding. Monoclonal antibodies 6E10, 11G5, 10F6, and 10D8 have previously been shown to exhibit virus neutralizing activity [15]. RCM, reduced and carboxymethylated.

Table 3. Comparison of antibody to recombinant gp120 from the IIIB strain of HIV-1 (IIIB-rgp120) in humans and chimpanzees at 2 weeks after the third immunization with IIIB-rgp120.

Species	Immunogen	No.	HIV-1_{IIIB} titers: gp120 $\log_{10}$	V3 $\log_{10}$	HIV-1 Neut*	Avidity†
Chimp	IIIB-	X247	4.85	2.70	299	100
	rsgp160	X261	4.85	2.93	183	52
	IIIB-	X262	5.27	3.32	1302	82
	rgp120	X265	4.55	3.55	455	84
Human	IIIB-	M17	4.92	2.96	49	68
	rgp120	M22	4.83	2.78	30	46
		M27	4.68	2.10	36	46
		M20	4.55	2.02	<16	38
		M26	4.75	2.46	39	42
Human	HIV-1+	Pool	5.05	2.76	–	–

*Neutralization titers represent 50% end-point values as calculated by log-linear intercalculation. †Avidity represents the precentage of antibody remaining bound to IIIB-rgp120 coated microtiter plates dissociation in 8 M urea (see Materials and methods).

Antibody binding to native and RCM IIIB-rgp120 was measured in the sera from human volunteers collected 2 weeks after the third immunization with IIIB-rgp120. Antibody binding to native IIIB-rgp120 was typically 3–4-fold greater than binding to RCM IIIB-rgp120 (Fig. 4). When the data from antisera collected 2 weeks after the third immunization were analyzed (Table 1), the ratio of the mean antibody

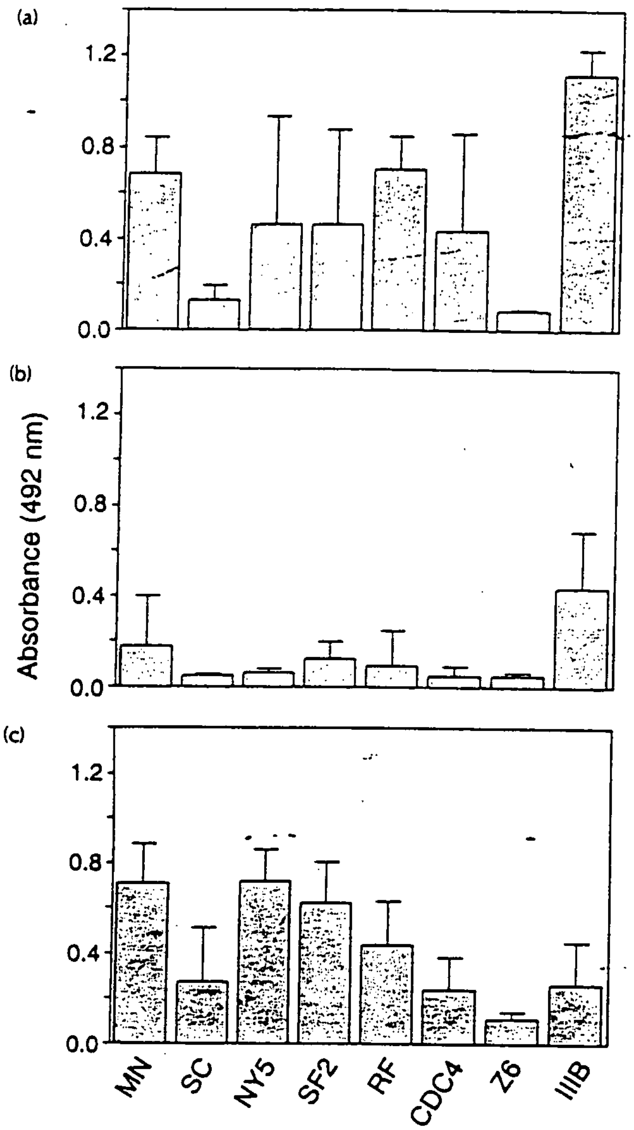

Fig. 5. Cross-reactivity of antisera from recombinant gp120 from the IIIB strain of HIV-1 (IIIB-rgp120) chimpanzees and humans with synthetic peptides corresponding to sequences from the V3 domains of gp120 from diverse isolates of HIV-1. (a) Antisera from chimpanzees (n = 2) and (b) humans (n = 10) that received three 300 μg doses of IIIB-rgp120 were assayed by enzyme-linked immunosorbent assay for the ability to bind to synthetic peptides corresponding to the V3 domains of gp120 from diverse isolates of HIV-1. (c) Sera from HIV-1-infected individuals (n = 5) is shown for purposes of comparison. Data represents mean optical density values measured at a single, arbitrarily selected serum dilutions for each group (i.e., 1 : 150 for chimpanzee antisera; 1 : 100 for antisera from IIIB-rgp120 immunized humans; 1 : 200 for antisera from HIV-1-infected humans). Error bars represent standard deviations. X-axes indicate the HIV-1 isolates from which the peptides were synthesized.

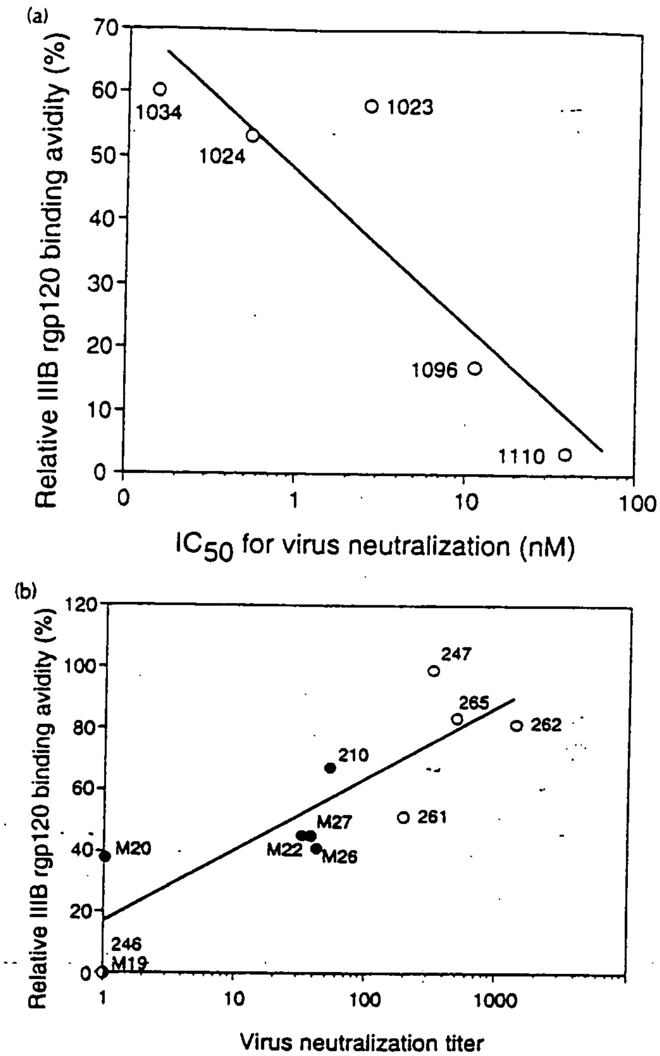

Fig. 6. Correlation between relative antibody binding avidity and virus neutralizing activity for monoclonal antibodies and polyclonal antisera to recombinant gp120 from the IIIB strain of HIV-1 (IIIB-rgp120). Relative antibody binding avidity was measured in an enzyme-linked immunosorbent assay where antibody binding to IIIB-rgp120 coated microtiter plates was measured before and after treatment with 8 M urea. Median inhibitory concentration (IC_{50}) values for virus neutralization of (a) monoclonal antibodies [15] to IIIB-rgp120 and (b) polyclonal sera from humans (M17, M22, M27, M20, and M26) and chimpanzees (262 and 265) that received three 300 μg immunization with IIIB-rgp120, or three 300 μg immunizations with IIIB-rsgp160 (247 and 261) were plotted as a function relative binding avidity. Serum from a placebo-immunized human volunteer (M19) and chimpanzee (246) is shown for purposes of comparison. ○, sera from chimpanzees; ●, sera from humans.

tion assay used in the present studies appeared less sensitive than that used by the AVEG Central Immunology Laboratory because the virus neutralization titers obtained were approximately 2–3-fold lower than those described previously [12].

Antibody avidity

In previous studies [19], we demonstrated that antibody binding affinity was an important correlate of *in vitro* virus neutralizing activity. In order to compare the relative binding affinities of the polyclonal antisera raised in humans and chimpanzees to IIIB-rgp120, an antibody binding avidity assay, similar to that described by Meurman *et al.* [17], was developed using IIIB-rgp120. In this assay, antibody

binding resistant to dissociation by 8 M urea was compared with antibody binding without a denaturant wash step. Preliminary experiments with MAb, whose 50% inhibitory concentrations (IC_{50}) for virus neutralization were known from previous studies [19], demonstrated that the relative avidity of binding, as measured by resistance to urea-induced antibody dissociation, correlated with the MAb IC_{50} values for virus neutralization (Fig. 6a). Thus, after the urea wash, 60% of the binding remained with MAb 1034 which possessed an IC_{50} of 0.15 nM, and only 3.3% of the binding remained for MAb 1110 that possessed an IC_{50} of 41 nM [19]. Similarly, the residual binding after the urea wash step for MAb 1024, 1023, and 1096, which possessed intermediate IC_{50} values of 0.53, 2.59, and 11.3 nM, respectively, were proportional to their binding avidity (Fig. 6a).

When sera collected 2 weeks after the third immunization from the IIIB-rgp120 immunized humans was compared with that of the IIIB-rgp120 and IIIB-rsgp160 immunized chimpanzees (Table 3, Fig. 6b), it was found that more than 80% of antibody binding remained after the urea wash in both of the IIIB-rgp120-immunized chimpanzees (262 and 265) and one of the IIIB-rsgp160-immunized animals (247). When the sera from the five volunteers previously identified as possessing the highest virus neutralization titers were examined, four out of the five exhibited 40–50% residual binding and one exhibited residual binding of 68% (Fig. 6b). These studies demonstrated that antibody binding avidity correlated with virus neutralizing activity, and that the relative IIIB-rgp120 antibody-binding avidity was greater in the antisera from the immunized chimpanzees than in that from the immunized humans.

Discussion

The present studies directly compared the antibody responses elicited by a prototypic HIV-1 vaccine, IIIB-rgp120, in humans and chimpanzees. Because only two chimpanzees received IIIB-rgp120, the differences in the immune responses between chimpanzees and humans reported in these studies apply only to individual animals and are not statistically significant. Antibodies raised in humans and chimpanzees exhibited a high degree of cross-reactivity with a panel of rgp120 from seven diverse HIV-1 isolates. The fact that antisera from the IIIB-rgp120 immunized chimpanzees and humans all exhibited strong binding to proteins that differed in sequence by as much as 35% from IIIB-rgp120 [20], suggested that immunodominant epitopes for humans and chimpanzees are conserved between diverse strains of HIV-1. Similarly, immunization with IIIB-rgp120 elicited antibodies able to inhibit gp120 binding to CD4 in both species. Antisera from both chimpanzees and humans were more effective in blocking the binding of the homologous IIIB-rgp120 protein to CD4 than they were in blocking the binding of the heterologous MN-rgp120 protein. Similar results have been reported previously for guinea pigs and rabbits immunized with IIIB-rgp120 [14], and suggest that antibodies to both common- and specific- CD4 blocking epitopes were elicited. The strain specificity in CD4 blocking activity might be attributable to sequence differences in the C4 domain of gp120 where a polymorphisms that prevent the binding of CD4 blocking MAb have been described recently [19]. Antibody binding to native and denatured gp120 was studied to determine the extent to which the antibody response is directed to conformation-dependent epitopes. The average ratio of antibody binding to native and RCM denatured IIIB-rgp120 was approximately 3.3 : 1 and 2.2 : 1 in immunized humans and chimpanzees, respectively. Thus, the antibody response elicited in humans to IIIB-rgp120, like that in HIV-1-infected individuals [21], was largely directed towards conformation-dependent epitopes.

As reported previously [12], a significant correlation was found between the magnitude of the antibody response to the V3 domain and virus neutralization. Thus, the chimpanzees that exhibited virus neutralization titers that were approximately 10-fold greater than those in humans, also exhibited V3 titers that were approximately 10-fold greater. These results are consistent with the possibility that antibodies to the V3 domain account for much of the neutralizing activity in these sera. Although the chimpanzee sera exhibited broader cross-reactivity with the V3 domain peptides from other isolates of HIV-1 (such as, MN, NY5, SF2, RF and CDC4) than the human sera, we cannot be certain that there is a significant difference in the magnitude or breadth of the V3 antibody response between humans and chimpanzees. These studies do, however, demonstrate that the antibody responses to the V3 domain in the two IIIB-rgp120-immunized chimpanzees protected from HIV-1 infection, were higher and more cross-reactive than the V3 domain antibody responses in the IIIB-rgp120-immunized humans.

It is now recognized that antibody binding affinity, as well as antibody concentration, define the threshold for virus neutralization [19]. Because conventional methods of measuring binding affinity (such as the Scatchard analysis) cannot be used to characterize binding of a large multivalent antigen by polyclonal antibodies, we measured the relative avidity of antibody binding using a method that measured the ability of chaotropic agents to dissociate antibody binding to gp120. The assay was validated by observations that the relative antibody binding avidity for MAb correlated with antibody binding affinity ($r = 0.81$). Using this assay, the relative avidity of gp120 binding by chimpanzee sera was found to be

higher than the relative avidity of sera from IIIB-rgp120-immunized humans. Thus, the low neutralizing activity in sera from IIIB-rgp120-immunized humans might result from low binding avidity as well as low concentrations of antibodies directed to the V3 domain.

A significant finding of this study was that the duration of the humoral response to IIIB-rgp120 was approximately four times longer in humans than in chimpanzees immunized with IIIB-rgp120 or IIIB-rsgp160. Previous studies of the antibody responses to recombinant envelope glycoproteins from the IIIB and SF-2 strains of HIV-1 in chimpanzees [2,3] and baboons [22,23] demonstrated that the antibody responses were short lived, with antibody titers falling approximately 100-fold 4–6 weeks after immunization. The present studies demonstrated that the duration of antibody response to IIIB-rgp120 in humans is longer than that seen in two species of primates (for example, chimpanzees and baboons) commonly used for preclinical immunogenicity studies. A significant difference between the duration of the antibody response in humans and non-human primates was described previously [24] in connection with the development of the inactivated poliovirus vaccine.

The lower than expected virus neutralizing titers elicited in humans immunized with IIIB-rgp120 might be related to the longer duration of their immune response. Previous studies in chimpanzees [2,3] and baboons [22] suggested that the interval between immunizations was more important than the immunogen dose for eliciting high levels of virus neutralizing antibodies. Data from both the chimpanzee and baboon studies suggested that optimal boosting occurred only after the antibody titers had fallen by approximately 100-fold. In theory, as the levels of vaccine antigen decline, and the immune response decays, there is a progressive selection for B cells bearing high affinity antigen receptors (i.e., cell-surface Ig). Accordingly, boosting an antibody response before it has decayed would expand a disproportionate number of B-cell clones with low affinity antigen receptors. The fact that human volunteers received the third injection of IIIB-rgp120 at a time when their anti-gp120 titers had fallen only 10-fold could have selected for a low affinity antibody response and might explain the lower than expected virus neutralizing titers. If this hypothesis is correct, then a longer interval between booster immunizations might allow for significantly higher affinity antibodies and higher neutralizing titers. Based on the decay curves for the anti-gp120 antibody responses, it appears that a 12-month interval would be required for antibodies to fall by a factor of 100 and that a 0, 1, and 12-month schedule might be required to achieve the maximal virus neutralizing titers with alum formulated IIIB-rgp120. While this schedule would be difficult, or impractical to implement in high-risk individuals participating in vaccine efficacy trials, it might be acceptable for a final product targeted at individuals (for example, preadolescents) where there is no imminent risk of HIV-1 infection.

Acknowledgement

We would like to acknowledge Art Ammann, Ann Marie Duliege, Thomas Twaddell, and the NIAID AIDS Vaccine Evaluation Network for their help in the design and implementation of the clinical trials, and Don Francis, Allan Izu, and Tim Gregory for critically reviewing this manuscript and for many helpful discussions.

References

1. Fultz PN: Immunization and challenge of chimpanzees with HIV-1. *AIDS Res Hum Retroviruses* 1992, 8:1517–1519.
2. Berman PW, Groopman JE, Gregory T, *et al.*: Human immunodeficiency virus type 1 challenge of chimpanzees immunized with recombinant envelope glycoprotein gp120. *Proc Natl Acad Sci USA* 1988, 85:5200–5204.
3. Berman PW, Gregory TJ, Riddle L, *et al.*: Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. *Nature* 1990, 345:622–625.
4. Hu S-L, Fultz PN, McClure HM, Eichberg JW, *et al.*: Effect of immunization with a vaccinia-HIV env recombinant on HIV infection of chimpanzees. *Nature* 1987, 328:721–723.
5. Arthur LO, Bess JW, Waters DJ, *et al.*: Challenge of chimpanzees (Pan troglodytes) immunized with human immunodeficiency virus envelope glycoprotein gp120. *J Virol* 1989, 63:5046–5053.
6. Girard M, Kieny M-P, Pinter A, *et al.*: Immunization of chimpanzees confers protection against challenge with human immunodeficiency virus. *Proc Natl Acad Sci USA* 1991, 88:542–546.
7. Fultz PN, McClure HM, Swenson RB, *et al.*: Persistent infection of chimpanzees with HTLV III/LAV: a potential model for acquired immunodeficiency syndrome. *J Virol* 1986, 58:116–124.
8. Watanabe M, Ringler DJ, Fultz PN, *et al.*: A chimpanzee passaged human immunodeficiency virus isolate is cytopathic for chimpanzee cells but does not induce disease. *J Virol* 1991, 65:3344–3348.
9. Fauci AS: The human immunodeficiency virus; infectivity and mechanisms of pathogenesis. *Science* 1988, 239:617–622.
10. Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, Gregory TJ: Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the Type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J Biol Chem* 1990, 265:10373–10382.
11. Lasky LA, Nakamura G, Smith DH, *et al.*: Delineation of a region of the human immunodeficiency virus gp120 glycoprotein critical for interaction with the CD4 receptor. *Cell* 1987, 50:975–985.
12. Schwartz DH, Gorse G, Clements ML, *et al.*: Induction of HIV-1 neutralizing and syncytium-inhibiting antibodies in uninfected recipients of HIV-1IIIB rgp120 subunit vaccine. *Lancet* 1993, 342:69–73.
13. Lasky LA, Groopman JE, Fennie CW, *et al.*: Neutralization of the AIDS retrovirus by antibodies to a recombinant envelope glycoprotein. *Science* 1986, 233:209–212.
14. Berman PW, Matthews TJ, Riddle L, *et al.*: Neutralization of multiple laboratory and clinical isolates of HIV-1 by antisera

raised against gp120 from the MN isolate of HIV-1. *J Virol* 1992, 7:4464–4469.

15. Nakamura GR, Byrn R, Rosenthal K, *et al.*: Monoclonal antibodies to the extracellular domain of HIV-1_{IIIB} gp160 that neutralize infectivity, block binding to CD4, and react with diverse isolates. *AIDS Res Hum Retroviruses* 1992, 8:1875–1885.
16. Capon DJ, Chamow SM, Mordenti J, *et al.*: Designing CD4 immunoadhesions for AIDS therapy. *Nature* 1989, 337:525–531.
17. Meurman O, Waris M, Hedman K: Immunoglobulin G antibody avidity in patients with respiratory syncytial virus infection. *J Clin Microbiol* 1992, 30:1479–1484.
18. Pawels R, Balzarini J, Baba M, *et al.*: Rapid and automated tetrazolium-based colorometric assay for the detection of anti-HIV compounds. *J Virol Methods* 1988, 20:309–312.
19. Nakamura GR, Byrn R, Wilkes DM, *et al.*: Strain specificity and binding affinity requirements of neutralizing monoclonal antibodies to the C4 domain of gp120 from human immunodeficiency virus type 1. *J Virol* 1993, 67:6179–6191.
20. Myers G, Rabson AB, Berzofsky JA, Smith TF, Wong-Staal F: *Human Retroviruses and AIDS. A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences.* Los Alamos: Los Alamos National Laboratory; 1992.
21. Moore JP, Ho DD: Antibodies to discontinuous or conformationally sensitive epitopes on the gp120 glycoprotein of human immunodeficiency virus type 1 are highly prevalent in sera of infected humans. *J Virol* 1993, 67:863–875.
22. Anderson KP, Lucas C, Hanson CV, *et al.*: Effect of dose and immunization schedule on immune response of baboons to recombinant glycoprotein gp120 of HIV-1. *J Infect Dis* 1989, 160:960–969.
23. Haigwood NL, Nara PL, Brooks E, *et al.*: Native but not denatured recombinant human immunodeficiency virus type 1 gp120 generates broad-spectrum neutralizing antibodies in baboons. *J Virol* 1992, 66:172–182.
24. Salk J: Vaccination against paralytic poliomyelitis: performance and prospects. *Am J Public Health* 1955, 45:575–596.